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The long term goal of this project is to develop a novel method to target infection of retroviral vectors *in vivo* utilizing mice expressing a retroviral receptor transgene (the Rous sarcoma virus receptor). Directed infection, and thus directed gene expression of cells expressing the viral receptor should provide a rapid and efficient method to test the mammary tumorigenic potential of genes in an animal model. Unlike the traditional method of testing gene function in transgenic mice, directed infection can be temporally controlled allowing assessment of differences in oncogenic potential at different stages of mammary gland development. Finally, multiple oncogenes can be introduced by co-infection allowing questions of synergy to be addressed. Toward this long term goal, we produced two transgenic mouse lines carrying the RSV receptor. However before characterization of receptor expression or in vivo targeting, both transgenic lines were lost. Considerable progress on development of viral vectors for use in this targeting system was achieved. Vectors and procedures for the production of high titer murine leukemia virus (RSV) pseudotypes were established. These MLV(RSV) vectors allow greater flexibility and capability compared to available RSV vectors and will be of great utility for directed infection of mammary epithelial cells in transgenic mice when the mice become available.

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INTRODUCTION

Presently, the most common way to analyze gene function in a particular cell type *in vivo* is to generate a new transgenic line for each gene under study - a costly and time consuming endeavor. The project which this grant funded utilized mice expressing a retroviral receptor transgene (the Rous sarcoma virus receptor) to target infection of retroviral vectors *in vivo*. This approach allows directed infection, and thus directed gene expression, of cells expressing the viral receptor. Our hypothesis was that this would provide a rapid and efficient method to test the mammary tumorigenic potential of genes in an animal model. An important difference between this approach and the more traditional method of testing gene function in transgenic mice is that infection, and thus gene expression, can be temporally controlled. This allows assessment of differences in oncogenic potential of genes at different stages of mammary gland development. In addition, by allowing the gene to be introduced after gland development has occurred, it avoids effect of the genes on early gland development and might therefore provide a more relevant model of tumorigenesis. In addition, use of newly described retroviral-based cDNA libraries may allow identification of novel genes that can participate in mammary tumorigenesis. Finally, it should be possible to introduce multiple oncogenes by co-infection thereby allowing questions of synergy between these genes to be addressed.

To begin development of a receptor targeted infection system that would be of utility for analyzing mammary tumorigenesis we proposed the following goals for this research project:
TASK 1, Generate transgenic mice expressing RSV receptor in mammary epithelia.
TASK 2, Characterize expression of transgene in mammary gland.
TASK 3, Develop retroviral vectors and protocols for infection mammary epithelia of transgenic animals.

In preparation for this project, we collaborated with Steve Hughes at NCI to produce mice carrying RSV receptor transgenes. Using a muscle specific alpha-actin promoter/tva construct, we established five mouse lines carrying this transgene. Characterization of these lines by Western blot analysis demonstrated that the receptor

was specifically expressed in several types of muscle. Although the level and pattern of expression in muscle vary for each of the transgenic lines, these experiments demonstrate that in general, the receptor can be efficiently expressed without deleterious effects. Furthermore, using mice carrying a ß-actin promoter/tva construct Dr. Hughes lab has demonstrated expression of Tva in numerous cell types including early embryonic cells. Together the results with these two different promoters suggested that expression of Tva in many different tissues and at numerous developmental periods was not detrimental. Therefore, it we felt it was reasonable to attempt making transgenic animals expressing Tva in the mammary gland.

In published experiments using the α-actin-tva transgenic mice we directly demonstrated targeted retroviral infection *in vivo* [1]. 2000-5000 infectious units of an RSV vector carrying the bacterial alkaline phosphatase gene (RCAS(A)-BAP) were injected into the thigh muscle of 5 day old mice. At d5 post-birth there is significant myogenesis occurring such that the myoblasts, if susceptible to RSV-A, should be good targets for infection. Controls for the experiments included injection of a subgroup E RCAS-BAP vector which should not utilize the subgroup A receptor and injection of non transgenic littermates. Infection was seen only when the subgroup A virus was injected into transgenic mice. Several hundred infected myoblasts or myotubes were spread throughout the muscle and infection did not seem to be localized at the injection site. Furthermore, by injecting avian cells expressing the RSV vectors rather than the virus stock, infection of the myoblasts was dramatically increased such that thousands of cells were infected. These experiments provided a proof of principal for our proposed project using Tva to target mammary cells for infection *in vivo.*

In the time since this grant started there has been significant progress using the tva transgene system for directed infection in vivo. Recent published reports demonstrate that glial cells in the brain are specifically infected by RSV vectors if these cells express the RSV receptor, Tva [2]. Indeed, the ability to direct infection of glial cells allowed development of a unique animal model for glioma [2-4]. In another, unpublished report, genes can be delivered specifically to megakaryocytes expressing Tva (A. Leavitt personal communication) providing a unique animal model for studying the effect of genes upon platelet development. Finally, as is discussed below, Dr. Yi Li in Harold Varmus' lab has recently produced mice that express Tva in the mammary

epithelium and has in preliminary experiments demonstrated introduction of marker genes into the mammary epithelia (Yi Li and H. Varmus personal communication). Together with the published results [1] from Steve Hughes lab using tva expressed under the control of a muscle-specific promoter, this data from several labs clearly demonstrates the utility of tva for directed infection of cells in vivo. Indeed, because of the number of labs now using this system a one day meeting on in vivo Tva-directed infection and expression has been held annually at NIH for the past two years.

BODY

Expression of Tva in a mammary epithelial cell line allows RSV infection.

Although we have demonstrated that Tva functions in a number of cultured cells and can infect muscle cells *in vivo* we wanted to test directly whether mammary epithelial cells could utilize the receptor to allow RSV entry. To address this question, and to determine if the plasmid to be used as the transgene was functional, an MMTV LTR-*tva* construct was introduced into a cultured mammary epithelial cell line, C57MG. The transfected cells were then challenged with an RSV vector carrying a ß-galactosidase marker. Expression of the receptor renders C57MG cells highly susceptible to RSV infection as judged by ß-gal staining. These experiments demonstrate that at least in cultured mammary cells there is no block to RSV infection if the receptor is expressed, and they suggest that *in vivo* RSV vectors should infect the mammary epithelial cells of MMTV LTR-tva transgenics.

MMTV LTR-tva Transgenic mice

The expression pattern of the MMTV LTR in mice has been thoroughly characterized. MMTV LTR constructs have been used extensively to construct transgenic mice for the purpose of expressing genes in the mammary gland [5-10]. Abundant expression in mammary epithelial cells is seen when this promoter is utilized. In addition to mammary gland expression, the MMTV LTR also promotes relatively high levels of expression of transgenes in the salivary gland and the testis.

We used a construct with the MMTV LTR in the same orientation and immediately upstream of the tva coding region to generate the transgenic mice. Based on previous studies using an identical arrangement of the MMTV LTR and other

transgenes, this construct should promote receptor expression in mammary epithelial cells. After pronuclear injection of the MMTV LTR-tva construct and Southern blot screening of offspring, three potential founder mice carrying the transgene were identified (two male and one female). These mice were bred and offspring were screened for the transgene by Southern blotting. Figure 1 shows a representative blot analyzing DNA isolated from tails of potential transgenic mice. The Pst I digested genomic DNA shows a characteristic fragment of 365 base pairs when hybridized with a labeled probe specific for the tva sequence (marked with arrow in Figure 1). From the three potential founders described above, two lines of mice carrying the MMTV-tva gene were established. These lines were named for the founder mice and were called lines #1 and #22.

One of the parental lines of the hybrid mouse line that is used to produce transgenic mice at the University of Pennsylvania transgenic facility has a high spontaneous mammary tumor rate. In contrast, the C57BL/6 line of mice has a low spontaneous mammary tumor incidence (less than 2%). Therefore, in preparation for use of these mice in tumorigenesis studies, we began the process of crossing our two transgenic lines with C57BL/6 mice. We crossed the transgenic mice with C57BL/6 for 3 generations in order to move the MMTV LTR-tva transgene into the C576 genetic background where the spontaneous mammary tumor rate is low. Unfortunately, while we were performing this backcross procedure an outbreak of mouse hepatitis virus occurred in our mouse colony. The MHV infection coupled with poor breeding of our C57BL-6 backcrossed transgenic animals (possibly due to a concurrent pinworm infection) caused both lines #1 and #22 to be lost. While we were having these problems, Harold Varmus (NIH) informed us that his lab was also interested in establishing mouse lines that express a tva transgene under control of the MMTV LTR and other mammary-specific promoters such as the whey acidic protein (WAP) promoter. Initially, we proposed to re-establish the MMTV LTR-tva transgenic lines in a new barrier facility at the University of Pennsylvania, however because establishment of these lines was well underway at NCI, and in order to avoid duplicating their efforts, we instead focused our efforts on developing new vectors for use with these mice. Development of these vectors which are based on MLV(RSV) pseudotypes will greatly enhance our ability to introduce genes into mammary epithelia (see below).

Characterization of transgene expression

Previous studies characterizing the expression pattern of the MMTV LTR suggest that the MMTV-tva transgene should be expressed mainly in the mammary epithelial cells of the mammary gland. In addition, we would expect that the MMTV LTR will be expressed in the salivary gland and testis. Expression of Tva in the α-actin-tva transgenic mice was readily detected by both analysis of messenger RNA using RNase protection and also by western blot analysis of protein from muscle tissue using an anti-Tva antibody [1]. We focused our tva expression analysis on attempting to detect Tva protein. For this analysis mammary glands (glands # 3 and 4) from transgenic and non-transgenic, pregnant and lactating females were isolated and frozen (-80) till used. To prepare protein from the glands, the tissues were homogenized in a cell lysis buffer containing SDS. The conditions used were identical to those we previously used successfully for analysis of Tva protein from the actin promoter-tva mice. In addition, we also used a cell lysis protocol employing the detergent Triton X-100 instead of SDS. Protein in the Triton lysates was either analyzed directly or precipitated with the lectin concanavalin A agarose (ConA) to concentrate glycoproteins before analysis (unpublished data from our lab demonstrates that Tva is heavily glycosylated and efficiently precipitates with ConA-agarose beads). Proteins isolated from mammary glands by SDS lysis, Triton lysis or Triton lysis and Con A precipitation were analyzed by SDS/PAGE and western blotting using a polyclonal antibody specific for Tva. In all these analysis we were unable to detect Tva protein in the mammary glands from MMTV LTR-tva mice.

To increase the sensitivity of the Tva expression analysis, we purified the polyclonal anti-Tva antiserum used to detect the receptor protein. A fusion protein consisting of maltose binding protein fused in frame to Tva (MBP-Tva) was produced and purified from E. coli. This protein was coupled to sepharose beads and used to purify a rabbit polyclonal anti-Tva antiserum. This purified antibody is highly reactive for Tva and has very low background on western blots or in immunoflourescence. Analysis of western blots of lysates or Con A precipitated glycoproteins from the MMTV LTR-tva mice using this highly purified antisera was again unable to detect expression of Tva.

Interestingly, a similar result has been found by Yi Li when 10 lines of mice expressing tva from various mammary specific promoters were analyzed (Yi Li personal communication). In contrast, Tva expression was readily detected when purified anti-Tva sera was employed to analyze transgenic mice expressing tva from a megakaryocyte-specific promoter (Andy Leavitt personal communication) suggesting that our inability to detect Tva represents low level expression in the MMTV LTR-tva mice.

Virus vectors and stocks

To date, avian vectors developed by Steve Hughes at NCI, based on the replication-competent Rous sarcoma virus, have been the only viruses used to introduce genes into transgenic mice expressing tva. The main advantage of the RSV-based vectors (so called RCAS vectors) is the high titer virus (~10^6-10^7/ml) that can be produced by avian cells infected with this virus. These high titers are achieved because RCAS is a replication-competent vector. Although RSV-based vectors are replication-competent in avian cells, RCAS cannot replicate in murine cells due to blocks in RNA transcript splicing and virion assembly. Additionally, because RSV is an exogenous virus in mice, RCAS cannot recombine with or be helped by endogenous murine retroviruses thereby preventing spread of the RCAS vector beyond the initial targeted tissue. Despite these obvious strengths, a major limitation of the RCAS vectors is the size insert which they can accommodate. These are gene replacement vectors in which the introduced gene replaces the 2 kb src gene of RSV, thus the total insert size is limited to approximately 2kb. This is smaller than many cDNA's which one would like to study in the mammary gland and importantly makes it impossible to produce vectors which carry a marker gene to follow infection as well as a genes whose function is under analysis.

Another major limitation of the replication-competent RSV vectors is that they encode the structural proteins of this virus. Therefore, when these vectors are introduced into adult mice they are likely to elicit an immune response, potentially resulting in clearance of infected cells carrying the transduced gene. In contrast, the defective MLV vectors do not encode any structural genes and the only exogenous genes one has to worry about are the genes under analysis. It is presently unclear

whether the concerns about immune responses to RCAS vectors are warranted, however given recent attempts to introduce genes in other replication-competent vectors (such as adenoviruses) it seems highly likely this will be the case [11-13].

Because of the limitations of the RSV vector system, we proposed to develop a transient system to produce high titer stocks of MLV viruses carrying the RSV EnvA protein. These viruses would allow directed infection of these viruses into cells expressing tva, but would not suffer the limitations of the RSV vectors. Many viruses will acquire the envelope protein of another virus through a process known as pseudotyping. The pseudotyped virus acquires the target cell specificity of the foreign viral glycoproteins. We have taken advantage of pseudotyping to generate murine leukemia retroviruses carrying the RSV envelope glycoproteins (so-called MLV(RSV) psuedotypes). One advantage of the MLV(RSV) system over replication-competent RSV vectors is that the simpler, defective MLV vector backbone accommodates much larger inserts (up to 7kb). Importantly, because the MLV system is widely used by numerous labs around the world to introduce genes into cells in culture, many of the genes one would wish to study in relation to mammary gland development or tumorigenesis have already been produced in MLV vectors and can be readily produced as MLV(RSV) pseudotypes using the protocols outlined below.

To fully take advantage of a transient MLV system described by Kingsman's laboratory [14] that allows production of high titer MLV vectors we needed to extensively modify one of the original vectors described for use in this system (pHIT110). The pHIT110 vector was quite large (9.1kb) and contained a G418 resistance gene which we decided to remove to avoid potential immune recognition of infected cells. To do this we constructed the vector pHIT110 poly. This vector is much smaller (5.1kb) and unlike pHIT110, it is derived entirely from known sequences. Both these features allow more rapid introduction of numerous genes into this vector. pHIT110 poly contains a strong cytomengalovirus immediate early enhancer-promoter fused to the R and unique 5' (U5) region of MLV to allow very high levels of transcription, an extended MLV packaging signal for efficient incorporation of the genomic RNA into virions, a polylinker region with ten unique sites for introducing genes of interest, and the complete 3' murine sarcoma virus (MSV) LTR for efficient expression of the transduced gene in the infected cells. To test this vector, we inserted

a nuclear localized β-gal gene into pHIT110 poly and produced MLV pseudotypes carrying the vesicular stomatitis virus glycoprotein (VSV-G). Infection studies using these viruses demonstrate that titers up to 1 X 10^7 IU/ml are obtained with this modified vector. These titers are equivalent to those seen with the original pHIT vector and demonstrate that the re-engineered MLV vector is functional. Thus we now have a versatile MLV vector that readily allows introduction of marker genes or oncogenes and permits production of very high titer stocks.

An important requirement for vectors that will be introduced into the mammary gland in vivo is that they allow injection of a significant inoculum of virus in a very small volume - i.e. that they have a very high titer. This requirement stems from the fact that only very small volumes can be injected directly into the gland without leakage of the inoculum (roughly 10- 50μl). To achieve the highest titers possible we developed and optimized a protocol to produce and concentrate the MLV viruses pseudotyped with the RSV envelope proteins. The original three plasmid transfection system described by Kingsman's lab to produce high titer MLV stocks [14] was modified to allow production of high titer MLV(RSV) pseudotypes by substituting a CMV promoter driven RSV subgroup A envelope construct for the MLV envelope plasmid. In addition, we extensively analyzed the parameters for transient production of the MLV(RSV) viruses from 293T cells by varying the ratio's of the three (RSV envelope, MLV core, and MLV vector) input plasmids, by varying the length and frequency of viral harvests, and by varying the concentration and timing of sodium butyrate used to boost expression of the CMV promoter plasmids. The optimized protocol utilizes 20μg of pCB6 EnvA (e.g. RSV envelope plasmid), 10μg of pHIT60 (MLV core plasmid) and 20μg of the pHITpoly vector transfected using calcium phosphate onto 4X10^6 293T cells plated the previous evening. The DNA is removed 8 hours later and the cells are induced with 15mM sodium butyrate 36 hours after the DNA was added. At 48 and 60 hours post transfection the media is harvested and either titer directly or stored at -80 till used. Analysis of the titer of the virus produced by this optimized protocol demonstrates that very high titers of the MLV(RSV) pseudotypes are produced (Table 1). by optimizing the MLV(RSV) production protocol we have been able to increase the titers of these pseudotypes from our initial experiments (~1X10^5/ml) ten-fold to nearly the level seen with an envelope known to pseudotype MLV very well (e.g. VSV-G) [15]. Importantly,

the titer of the MLV(RSV) virus is now at a concentration that it is useful for in vivo injections.

To allow further increases in the titer of the virus stocks, we experimented with two procedures to produce high titer stocks, ultracentrifugation and ultrafiltration. We find that a high level of concentration can be achieved for EnvA pseudotyped viruses by ultracentrifugation while in our hands ultrafiltration results in a loss of infectivity. It appears that the most critical parameter for successful concentration by ultracentrifugation is slow resuspension of the pelleted virions (overnight at 4° without vortexing works best). Thus, the protocol routinely employed to concentrate MLV(EnvA) viruses is to harvest media from transiently transfected 293T cells 36 hours after the DNA is added. The media is clarified by low speed centrifugation (3600 rpm for 10 minutes) and then virions are concentrated by centrifugation at 80K X g for 15 minutes. The viral pellet is resuspended in Tris buffered saline overnight at 4°. Using two sequential concentration steps we have achieved a greater than 200-fold increase in MLV(EnvA) β-gal titers as assayed on cultured cells (average final titer $2X10^8$IU/ml on quail QT6 cells). A viral stock of this titer allows sufficient virus in 25μl to inject of 5 X 10^6 IU of cell-free virus per gland.

Mammary glands taken from mice during lactation or immediately post-weaning appear to contain significant levels of endogenous β-gal activity which masks detection of infected cells using a β-gal marker gene (see below). To avoid problems detecting a β-gal marker gene in this setting, we constructed vectors that carry markers other than β-gal for analysis of *in vivo* infection. One such vector is an MLV genome encoding green fluorescent protein (GFP) or variants of GFP that have been optimized for expression in eukaryotic cells or that have shifted excitation and emission spectra compared to wild type GFP. To avoid potential problems of in vivo immune responses to other proteins encoded by the MLV vectors, we are using the minimal viral vector described above (pHIT poly) that encodes GFP (pHIT-GFP) but no additional selectable marker gene. In addition, we have obtained a vector encoding a human alkaline phosphatase from Dusty Miller (pLNCAP). This vector encodes G418 resistance as well as alkaline phosphatase. We are currently moving the AP gene of pLNCAP into pHIT110 poly to produce pHIT-AP which should avoid immune response problems

mentioned above and from our experience should result in higher titer MLV(EnvA) viruses.

The oncogene wnt-1 (initially called int-1) was initially identified during analysis of proviral insertion sites in MMTV-induced tumors. Subsequently a family of genes related to int-1 and the drosophila analog, wingless, were discovered. This family of genes (Wnt genes) are important mediators involved in morphogenesis. To analyze the function of Wnt genes other than wnt-1 in mammary tumorigenesis and development we have obtained a collection of seven murine wnt genes from Jan Kitajewski (Columbia University). The encoded Wnt proteins contain a C-terminal HA epitope tag that should allow the introduced proteins to be differentiated from endogenous murine Wnt's. These genes were cloned into the MLV vector LNCX and have been used to generate MLV(EnvA) pseudotypes by transient transfection of 293T cells. Titters ranging from $3X10^5$ to $8X10^5$ G418 resistant colonies per ml have been obtained. We are collaborating with Dr. Yi Li of the Varmus lab to assess the ability of these wnt genes to induce mammary tumors in MMTV-tva and WAP-tva transgenic mice.

One of the long term goals of this project is to identify novel genes which have the capability of inducing mammary tumorigenesis. Toward this end, we have developed MLV-based vectors that carry cDNA libraries from a variety of sources. We obtained MLV vector-based cDNA libraries from transformed murine early hematopoetic cells lines from Toshio Kitamura (University of Tokyo) [16]. In addition, we produced retroviral-based libraries from transformed avian (QT6) and human (HeLa) cell lines. We are in the process of making retroviral-based libraries from a murine mammary epithelial cell line, C57 MG. These libraries have been produced either in the original vector described by Kitamura which lacks a selectable marker or more recently in a bicistronic vector in which the cDNA is inserted upstream of an internal ribosome entry site (IRES) and GFP marker gene. These libraries will be used to identify genes that can participate in mammary tumorigenesis

Infection of mammary gland cells *in vivo*

To begin addressing whether Tva can efficiently direct infection of mammary epithelial cells in mice, we attempted to determine if viruses carrying the RSV envelope glycoprotein can infect mammary cells in the MMTV LTR-tva transgenic mice. As

discussed above we were unable to demonstrate that the mice express Tva, however we decided to proceed with the infection experiments since characterization of Tva expression in avian cells and in some mammalian cells transfected with tva constructs clearly demonstrates that extremely low levels of the receptor can mediate efficient infection [18, 19, and P Bates unpublished obsevation]. Therefore, it is possible that we may not detect expression of the tva transgene, yet the cells in the mammary gland of the transgenic mice will be infectable by subgroup A RSV enveloped viruses.

The pilot infection experiments in the MMTV-tva transgenics were performed on female mice that were pregnant (17 days) to ensure that there would be actively replicating mammary epithelial cells since RSV vectors require mitosis for integration and expression of the provirus. Mice were injected with either MLV(RSV) pseudotyped virus harvested from transiently transfected 293T cells or with the transfected 293T cells themselves. We had previously demonstrated that injection of avian cells expressing RSV vectors gave significantly better infection of muscle in the α actin-tva mice than injection of cell-free virus [1].In addition, in those studies it was demonstrated that the injected avian cells were rapidly cleared by the immune system (within 2-3 days), thus we did not expect that the injected 293T cells would persist and give misleading β-gal activity. To analyze Tva-directed infection in the MMTV LTR-tva transgenic mice, pregnant transgenic females from line #22 (due to limited number of mice only one mouse from line #1 was analyzed) were injected with 10^5 infectious units of an MLV(RSV) virus encoding β-galactosidase or with 10^7 293T cells transfected to produce the same virus. Cells or cell-free virus was injected directly into mammary glands #3 or #4 respectively in MMTV LTR-tva transgenic mice. Ten days after injection the mice were sacrificed and the glands were harvested. Whole mounts of the glands were prepared and stained for β-galactosidase activity.

Betagalactosidase activity in mammary glands infected with the MLV(RSV) viruses was readily detected in all the injected transgenic animals. While these experiments initially suggested that the MMTV LTR-tva mice had been infected with an MLV(RSV) virus that carried a β-galactosidase reporter gene, further analysis of additional mice infected with MLV(RSV) β-gal viruses demonstrates that this conclusion may have been premature. In repeating this work, we found that while there is significant β-gal activity detected in the infected glands, glands from uninfected animals

also display significant staining, suggesting that there is a high level of endogenous β-gal activity in the lactating mammary gland. To confirm this, we injected virus into the right #4 and #3 glands in a pregnant MMTV LTR-tva transgenic mouse while the left glands in these animals were not injected. Three weeks later all four glands were harvested and stained for β-gal activity. When infected and uninfected glands from the same animal were compared, no difference in the level of staining was seen (Figure 3). This finding suggests that the β-gal staining we observed is likely due to endogenous enzyme activity in the mammary gland either because of lactation or during regression following lactation. Thus any infection of the β-gal marked viruses would have been obscured by the high endogenous activity. About this time the animal husbandry problems discussed above caused the loss of these lines abrogating any further attempts to analyze infection by the MLV(RSV) vectors. Therefore, we cannot make any firm conclusions about the infection of our mice by the MLV(RSV) vectors. However, the group at NCI has recently documented efficient infection of mammary epithelial cells in the new MMTV LTR-tva transgenic lines which they have generated (Yi Li personal communication). In contrast to our experiments, they injected an alkaline phosphatase (AP) encoding RSV vector into glands at 5 weeks and analyzed AP expression 2-4 weeks after infection. Their result is clearly demonstrates that Tva functions in vivo to direct retroviral infection and validates our original hypothesis.

CONCLUSIONS

This grant was a career development award to Dr. Paul Bates to promote his research in the field of breast cancer. A goal of this grant was to foster development of a unique animal model in which genes could be rapidly and efficiently introduced into mammary epithelial cells in vivo. Development of this model required two components; first mice expressing a retroviral receptor in mammary epithelia cells and secondly retroviral vectors which could deliver the genes to be analyzed. During the tenure of this grant we have made significant progress on both these fronts.

In the initial years of this project we produced a transgene construct containing a mammary epithelial cell-specific promoter (the MMTV-LTR) driving expression of a cDNA encoding the cellular receptor for the avian subgroup A Rous sarcoma virus, tva. Analysis of the MMTV LTR-tva construct in stably transfected C57MG cells

demonstrated that the receptor protein was expressed from the MMTV LTR. Furthermore, the C57MG lines expressing Tva were susceptible to RSV(A) infection confirming the function of the transgene construct.

The MMTV LTR-tva construct was utilized to produce transgenic mice using standard pronuclear injection protocol. Three potential founder mice carrying the transgene were identified from the 52 offspring screened by Southern blot analysis for the transgene. From these three potential founders two lines carrying the transgene were established. Our ability to establish these lines, coupled with the frequency of potential founders, suggested that the MMTV LTR-tva transgene was not lethal or toxic. The fact that the transgene was tolerated was expected since we had previously collaborated with Dr. Steve Hughes to produce mice that express tva in muscle and had seen no deleterious affects of the transgene [1]. Furthermore, a subsequent reports in which tva expression was driven by a beta actin [1 7] promoter that is active in numerous tissues or by a glial cell-specific promoter [2] also suggested that tva transgenes are well tolerated. Finally, unpublished results from Dr. Harold Varmus' lab demonstrating production of 10 transgenic lines in which tva expression is driven using the MMTV LTR and other mammary-specific promoters directly supporting our conclusion that the MMTV LTR-tva transgene is not toxic in vivo.

Our efforts to demonstrate expression of Tva in the mammary gland were however unsuccessful. Western blot analysis using either crude or highly purified Tva-specific antisera failed to detect protein expression in mammary glands of MMTV-LTR-tva mice. Similarly, western blot analysis of glycoproteins from the mammary gland after precipitation with concanavalin A beads also did not allow Tva detection. This is in contrast to our analysis of the tva transgene expressed in muscle where Tva was readily detected [1]. Since our lab has significant experience analyzing Tva expression our inability to detect Tva in cells from mammary glands of the MMTV LTR-tva transgenics is not likely a technical problem and suggests that these two transgenic lines do not express appreciable levels of receptor, perhaps implying that high levels of Tva are not tolerated in the mammary gland. Interestingly in their analysis of several other transgenic mice expressing tva from mammary cell-specific promoters, the Varmus lab has also had difficulty detecting tva expression (Yi Li and H. Varmus, personal communication).

Unfortunately, during our analysis of the MMTV LTR-tva transgenic lines there was a husbandry problem that resulted in the loss of both of the mouse lines. Initially, we proposed to re-establish the MMTV LTR-tva transgenic lines in a new barrier facility at the University of Pennsylvania. However, discussions with Dr. Harold Varmus' laboratory at the National Cancer Institute prompted us to alter our plans. The Varmus lab had independently established several mouse lines that express a tva transgene under control of the MMTV LTR or other mammary-specific promoters such as the whey acidic protein (WAP) promoter. Because these lines were being produced at NCI, and in order to avoid duplicating their efforts, we have instead focused our efforts in the last year of this grant on developing new vectors that will greatly enhance our ability to introduce genes into mammary epithelia. We will use these vectors with the transgenic lines developed at NCI.

Indeed it is development of the vectors to use in gene delivery that we have made the most significant progress in the last two years of this proposal. We have developed and optimized a protocol for production of MLV(RSV) pseudotypes that allows generation of viral stocks with titers at or above 10^6 per ml. Because injection of virus in vivo requires that very low volumes be employed, we have also optimized procedures for ultracentrifugal concentration of the MLV(RSV) stocks that allow a greater than 200 fold concentration producing MLV(RSV) β-gal viruses with a titer of $>10^8$ per ml. The ability to use murine leukemia virus based vectors is of critical importance to the long term utility of this system for introduction of genes in vivo. The MLV vectors provide the capability to insert genes that are too big for the RSV vectors or to introduce markers genes along with the gene of interest. In addition, coupling the MLV(RSV) system with recently developed MLV-based cDNA libraries may allow identification of new genes involved in mammary tumorigenesis.

In addition to developing the protocols for efficient pseudotype production, we have also constructed MLV-based expression vectors to use to introduce genes into these mice. A modified MLV vector, pHIT110 poly, has also been constructed (Fig. 2). This vector deletes all protein encoding sequences to avoid any potential problems of immune response to the selectable markers. pHIT poly retains a chimeric 5' LTR that utilizes the CMV promoter enhancer for high level expression of the MLV genomic RNA and has an extended packaging sequence for efficient incorporation during transient

production of virus. Experiments with a derivative of pHI110 poly encoding β-gal demonstrated that this vector works well for production of high titer stocks. Finally, in preparation for studies looking at tumorigenesis in the mammary gland we have constructed MLV-based vectors carrying either potential oncogenes (murine Wnt's) or cDNA libraries. Indeed we are extremely excited at the prospect of using the retroviral-based cDNA libraries in conjunction with targeted infection of mammary epithelial cells to search for new genes that have the potential to induce mammary neoplasia.

Table 1. Titer of MLV(RSV) pseudotype viruses.

	Titer on Target cells[b]	
Viral Envelope[a]	**NIH3T3**	**3T3Tva**
none	0	0
EnvA	0	0.5-1 X 10^6
VSV-G	1-5 X10^6	1-5 X10^6

[a] The MLV virus was produced by transient transfection of 293T cells with plasmids HIT60 (expressing MLV gag-pol) and HIT111 (MLV genomic vector carrying β-galactosidase marker gene) essentially as described (14). To produced pseudotyped virus, plasmids encoding either RSV EnvA or vesicular stomatitis virus glycoprotein (VSV-G) under control of the CMV promoter was co-transfected with HIT 60 and HIT111.

[b] Viral titers on NIH3T3 or NIH3T3 target cells expressing Tva were determined by infecting the target cells with dilutions of media harvested from the transiently transfected 293T cells. After an overnight infection, infected cells were grown for 48 hours then fixed and stained for β-galactosidase activity. Titer is expressed as number of β-gal positive foci per milliliter of transfected cell supernatant. Range of titer observed in several independent experiments is given.

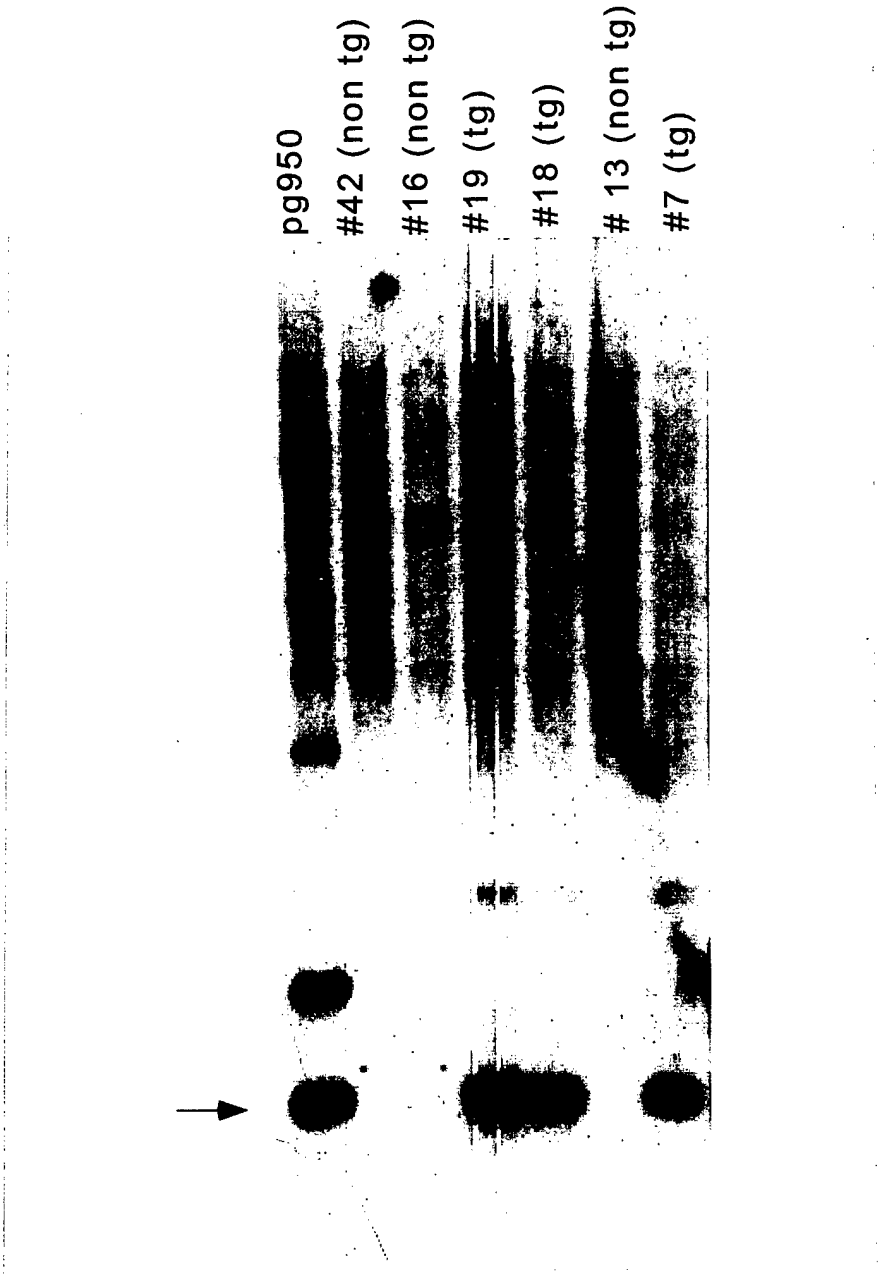

Figure 1. Southern blot analysis of the MMTV-tva transgene in DNA of line #22 offspring. Genomic DNA (10 µg) prepared from tails of 10-15d pups was analyzed for the MMTV-tva transgene. After digestion with Pst I, separation on a 1.5% agarose gel and transfer to nylon, the blot was hybridized with a random prime labeled probe specific for tva. The arrow indicates a 365 base pair band expected for the tva transgene. The pg950 lane contains a control DNA sample from the 3T3Tva cell line stably transfected with a Tva expression vector. The numbers above each lane indicate different offspring (F2 and F3 generations) derived from the #22 founder animal.

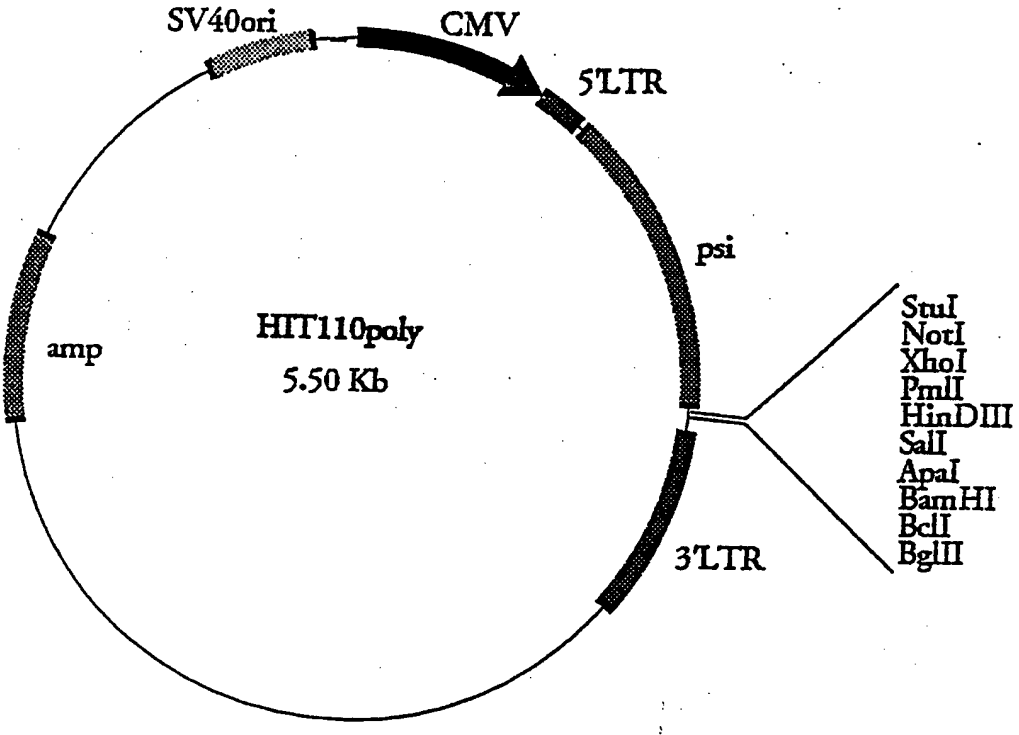

Figure 2. Map of pHIT110 poly MLV vector. A modified vector for transient production of viruses from 293T cells was constructed. The chimeric CMV promoter-enhancer-5'LTR from pHIT110 was cloned into the plasmid vector pSP72 (Promega), then the MSV 3' LTR and polylinker sequences from pSL1180 (Pharmacia) were inserted to produce pHIT110 poly.

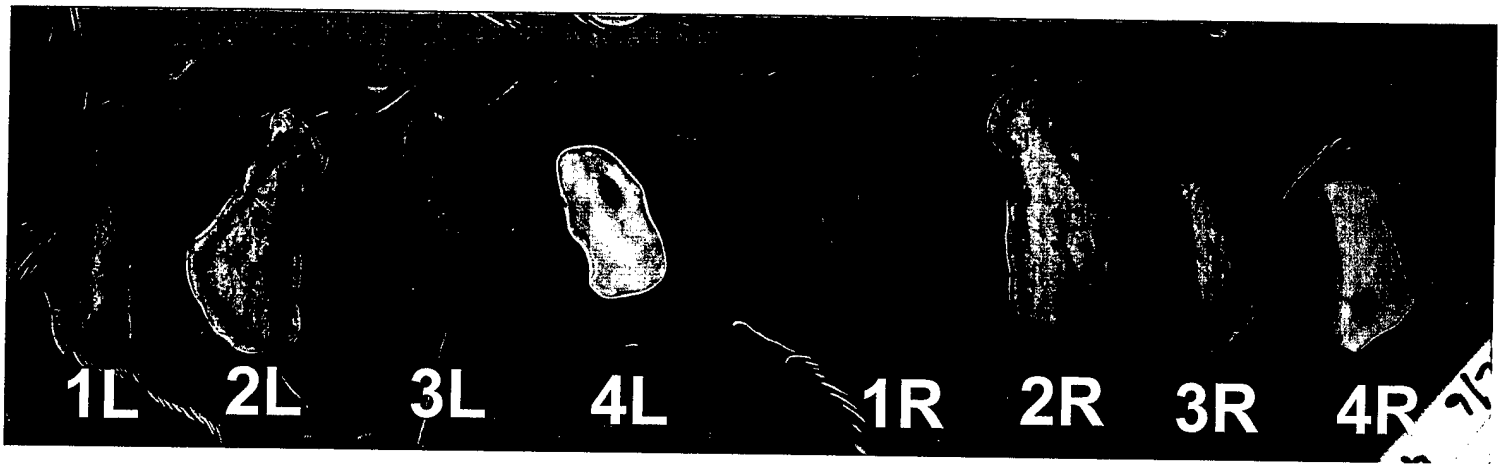

Figure 3. Betagalactosidase activity in lactating mammary glands. The right #4 gland of three mice was injected at approximately day 17 of pregnancy with either cell free MLV(EnvA) β-gal virus (3R), 293T cells producing this virus (1R) or mock injected (2R). The left gland was kept as an uninfected control (1L, 2L, 3L). Three weeks later the mice were euthanized and the glands mounted on slides and stained as whole mounts for β-gal activity. Both right and left glands were harvested from an uninjected, non-lactating 10 week old mouse (4L and 4R). As can been seen, the level of β-gal staining is independent of the injected virus and seems to be specific for the state of the gland in each mouse.

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